PATENT SPECIFICATION

872,536

DRAWINGS ATTACHED.

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COMPLETE SPECIFICATION.

Blood Coagulation Components and Method of Concentrating and Purifying.

We, CUTTER LABORATORIES, a Corporation organised under the laws of the State of California, United States of America, of City of Berkeley, State of California, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to blood coagulation components and provides a purified composition of blood coagulation components and a method for producing such a composition.

The blood coagulation mechanism is extremely complicated and involves the interaction of at least 19 or 20 different protein substances, including plasma thromboplastin component, proconvertin, and prothrombin. Although the coagulation mechanism is conveniently diagrammed in Albritton's Standard Values in Blood, pages 11 to 15 (W.B. Saunders Co., 1952), Albritton's diagrams do not show the role played by plasma thromboplastin component, for this component has been discovered only recently.

Plasma thromboplastin component is a component which is missing from the blood of all patients afflicted with haemophilia B, and is essential for the formation of plasma thromboplastin. The other thromboplastin precursors are thromboplastinogen (missing from the blood of haemophilia A patients) and a platelet factor. Proconvertin is a component which is essential for the rapid conversion of prothrombin to thrombin. Prothrombin is the actual precursor of thrombin.

[*Price* 3*s.* 6*d.*]

Because of the primitive state of knowledge concerning blood coagulation, confusion may result from variations in nomenclature, and in attempting to avoid this it should be noted that plasma thromboplastin component is also known as Plasma Thromboplastin Factor-B, and Christmas Factor, and that proconvertin is also known as Stable Component, Factor VII (Kohler), and Serum Prothrombin Conversion Accelerator Precursor.

There are approximately 60,000 cases of congenital haemophilia in the United States. Of these, approximately 9,000 are cases of haemophilia B, the blood of such patients being either totally devoid of plasma thromboplastin component or seriously deficient in plasma thromboplastin component. The disease therefore exists in varying degrees of severity, requiring therapy from once every three weeks up to once or twice a year. The completely deficient cases require replacement therapy once every three weeks; the partially deficient cases, which are the majority of cases, require therapy only when bleeding episodes occur, which may be as seldom as once a year. The bleeding episodes in congenital, partially-deficient cases are generally caused by a temporarily acquired severe deficiency rather than by injury alone. Intravenous injection of approximately 800 ml. of fresh plasma, or an equivalent amount (about 1200 ml.) of fresh blood, temporarily corrects the defect of a completely deficient subject. The beneficial effect lasts for about three weeks, although the coagulation defect, as measured by *in vitro* tests on the patient's blood appears normal for only three or four days.

Such therapy with fresh plasma or fresh blood is effective but it has two important drawbacks: (1) is requires ready availability of a large amount f fresh bl od and requires hospitalization for the administration of the blood; (2) a great many of the patients eventually become sensitized to repeated blood infusions and ultimately encounter fatal transfusion reactions.

There are estimated to be about 400 patients with congenital proconvertin deficiency still living in the United States. These are serious cases, and the patients seldom survive beyond childhood. Intravenous injection of approximately 300 ml. of fresh plasma, or an equivalent amount (about 500 ml.) of fresh blood, temporarily corrects the proconvertin deficiency in a child. Repeated therapy of proconvertin deficiency with plasma or blood is subject to the same costs and hazards as pointed out above in connection with plasma thromboplastin component deficiency. Proconvertin deficiency is also temporarily acquired during some liver infections, during some transfusion reactions, and from overdosage with dicumarol, and if prompt treatment with an available therapeutic agent were possible, it would be life-saving in many cases.

At the present time we do not know of any incidence of congenital prothrombin deficiency. However, temporary prothrombin deficiencies are acquired in the same manner as the temporary proconvertin deficiencies. The infusion of fresh blood or plasma into patients does not produce any measurable increase in the prothrombin blood level.

More specifically, one of the objects of this invention is the provision of a synergistic mixture of human blood coagulation components, including plasma thromboplastin component, proconvertin, and prothrombin, and which may be used far more effectively than fresh blood or plasma.

The present invention provides a derivative of human blood comprising plasma thromboplastin component free of other thromboplastin precursors.

It is also within the purview of this invention to provide the process of collectively recovering the proteins: plasma thromboplastin component, proconvertin, and prothrombin and non-citrated human plasma, comprising: mixing said plasma with an adsorbing agent such as barium sulphate, separating said adsorbing agent and its adsorbed proteins from the plasma, eluting the adsorbed proteins from said adsorbing agent with a sodium citrate solution, adjusting the *p*H of the solution to about 3.2, precipitating the proteins with a lower aliphatic alcohol e.g. ethanol, and collecting the resulting precipitate.

In the drawings appended hereto and made a part hereof:—

Fig. 1 includes a pair of curves illustrating the effect on a haemophilia B patient of the administration of various amounts of plasma as compared with the administration of various amounts of the product of this invention.

Fig. 2 includes a pair of curves illustrating the effect on the prothrombin consumption and prothrombin plus proconvertin consumption of a haemophilia B patient following the injection of 1120 ml. of fresh plasma and following the injection of 115 mg. of the product (No. 1141) of this invention.

Fig. 3 includes three curves illustrating the comparative effects on the clotting time in a haemophilia B patient resulting from the administration of 560 ml. of fresh plasma, 1120 ml. of fresh plasma, and 150 mg. of the product No. 1141 of this invention.

Details of Process.

By way of illustration, the process above referred to can be carried out as follows:—

1. Collect blood into an anticoagulant solution containing 750 mg. disodium ethylene diamine tetra-acetate in 20 ml. water for every 500 ml. blood. The red and white cells are separated by centrifuging and discarded.

2. Adjust 100 litres of the plasma to *p*H 6.0 and add 4 kg. of finely powdered barium sulphate. After stirring for approximately 30 minutes, separate the plasma from the barium sulphate. The plasma can then be fractionated into its other clinically useful proteins by conventional means.

3. Wash the barium sulphate with 100 litres of 0.9 per cent sodium chloride.

4. Elute a fraction of the adsorbed proteins from the barium sulphate using about 100 litres of 4 per cent sodium citrate adjusted to *p*H 7.5. Discard the barium sulphate.

5. Adjust the eluate to *p*H 7.0, add 15 per cent ethanol, at −5° C., and discard the precipitate which forms.

6. Adjust the supernatant solution to *p*H 3.2, add 15 per cent ethanol, at −5° C., and collect the precipitate which forms.

7. Lyophilize the precipitate. The resultant dry weight is about 20 grams.

8. Dissolve the precipitate in about 4.0 litres of aqueous diluent and remove and discard insoluble impurities.

9. Sterilize the solution by filtration through a bacteria-excluding filter, and aseptically fill 40 ml. quantities into serile vials.

10. Lyophilize the contents of each vial. The contents of each vial should be redissolved in sterile water just prior to use. The coagulation components are obtained in an over-all yield of 65 per cent or better.

The above process recites the details which are preferred at this time. However, many variations can be made without departing from the general principles of the process. Some variations have been tried, and the resultant products were subjected to clinical test in human subjects. This type of human testing is so difficult and so hazardous to the patient, since the patient must be allowed to reach an incoagulable state prior to the test, that it is impracticable to test the effects of all reasonable variations in process. Some possible variations have been established, however.

For example, instead of the disodium ethylene-diamine tetra-acetate anticoagulant one can substitute a sodium oxalate anticoagulant, or during the blood collection the blood can be treated immediately with a calcium-binding ion exchange resin such as "Dowex" 50, a sulphonic acid styrene type resin manufactured by the Dow Chemical Company. The conventional Anticoagulant Sodium Citrate Solutions, USP, or Anticoagulant Acid Citrate Dextrose Solution, USP, cannot be employed as anticoagulants if one wishes to obtain purified plasma thromboplastin component by the process of this invention. If a citrate anticoagulant is used, the final product contains the usual amount of prothrombin and proconvertin but is almost totally lacking in *in vivo* plasma thromboplastin component activity. This has been demonstrated repeatedly.

The *p*H of step No. 2 can be varied considerably, at least between *p*H 5.4 and 7.2, but the highest purity along with adequate yield of plasma thromboplastin component and proconvertin is obtained when this step is conducted near *p*H 6.0.

Other adsorbents can be substituted for the barium sulphate. For example, aluminium hydroxide or asbestos can be used. Larger or small amounts of adsorbents can be used. The use of appreciably smaller amounts of adsorbent causes a lower yield of the coagulation components; the use of too large amounts of adsorbent causes a loss of yield of other valuable products of plasma fractionation such as fibrinogen, gamma globulin and albumin. Here it should be noted that the action of these adsorbing agents is physical rather than chemical.

In step No. 4, any non-toxic electrolyte may be substituted for the sodium citrate, for example, sodium phosphate, if present in a high enough concentration to effect the elution. If the *p*H at step No. 4 is too high, the coagulation components are gradually destroyed. If the *p*H is too low, elution is slow and an excessive volume of eluting fluid is required. With these limitations in mind, the coagulation components can be eluted to a useful degree at *p*H values between 6.5 and 8.0.

Step No. 5 is optional. It effects a higher degree of purification by the removal of some impurities. If one omits step No. 5 and goes directly to step No. 6, th final product is useful clinically but is of somewhat lower purity. If the *p*H of step No. 6 is below 3.0, there is a progressive loss of prothrombin activity. As the *p*H is increased above about 4.5, there is a progressive loss of yield of plasma thromboplastin component and proconvertin. Although prothrombin can be recovered quantitatively up to *p*H 5.6, a *p*H in the region of 3.2 is preferred. The ethanol concentration at step No. 6 can be increased of course, but nothing is gained by doing so. As water-miscible volatile protein precipitants in steps 5 and 6, only ethanol, methanol, and isopropanol have been tried; all three were satisfactory.

Step No. 7 is optional. However, following resolution, a small amount of impurity precipitates and it is desirable to remove this prior to the sterilizing filtration.

The aqueous diluent in step No. 8 can be anything which prevents the adsorption of the coagulation components on the bacteria-excluding filter. Such aqueous diluents as a 2.5 per cent solution of serum albumin, diluted "barium sulphate-adsorbed" blood serum, and 0.3 molar glycine, have been used successfully.

The step of lyophilization in the final containers is also optional. The final product is stable as a frozen solution as well as stable in the dry state. It gradualy deteriorates with time when in solution, and it is preferred to distribute the product in the dry or frozen state.

Character of Product.

At all events, the product resulting from the process above described consists of a small amount of sterile, dry powder including a mixture of highly purified plasma thromboplastin component, proconvertin, and prothrombin. 150 mg. of the product when dissolved in a small quantity of sterile water contains the plasma thromboplastin component, proconvertin, and prothrombin originally present in 800 ml. of fresh plasma or 1200 ml. of fresh blood. The product contains less than 0.3 per cent of the total plasma proteins.

Testing Limitations.

There is as yet no reliable method for estimating the potency of the plasma thromboplastin component *in vitro*. Prothrombin or proconvertin or prothrombin plus proconvertin can be assayed reliably *in vitro*, but for assessing the potenency of a preparation of plasma thromboplastin component it is necessary to rely on the actual intravenous administration of the preparation into

a completely-deficient patient and then follow the patient's clotting time, per cent prothrombin consumption, and thromboplastin generation for two weeks. The proconvertin content can be assayed by th method of Owren and Aas, Scand. J. Clin. Lab. Invest. *3*, 201 (1951). The prothrombin content can be assayed by the method of Kjort, Rapaport, and Owren, J. Lab. Clin. Med *46*, 86 (1955). The combined proconvertin plus prothrombin can be assayed by the method of Ware and Stragnell, Am. J. Clin. Pathol. *22*, 791 (1952). In spite of optimistic reports in the literature which have never been verified clinically, the potenency of purified plasma thromboplastin component as assayed *in vitro* by either the conventional Prothrombin Consumption Test or the conventional Thromboplastin Generation Test bears no relationship to the clinical effectiveness of the purified plasma thromboplastin component in human subjects.

Clinical Results.

In patients congenitally afflicted with haemophilia B and completely deficient in plasma thromboplastin component, the intravenous injection of a solution of 150 mg. of the product of this invention corrected their coagulation defect for a period of 14 days and maintained the patients symptom-free for varying periods in excess of three weeks.

For example, Fig. 1 compares the immediate effect on the clotting time of a haemophilia B patient following the injection of varying amount of fresh plasma and following the injection of varying amounts of a solution of the product (No. 1141) of this invention. Each injection and measurement was made following an interval of at least two weeks, during which period no treatment was given.

Figure 2 compares the prolonged effect on prothrombin consumption of a haemophilia B patient following the injection of 1120 ml. of fresh plasma and following the injection of a solution of 150 mg. of product No. 1141. In the prothrombin consumption test, the lower the per cent prothrombin, the more effective is the agent in converting prothrombin to thrombin *in vivo*.

Figure 3 compares the prolonged effect on clotting time in a haemophilia B patient following the injection of 560 ml. of ACD fresh plasma, 1120 ml. of ACD fresh plasma, and a solution of 150 mg. of product No. 1141.

As the above tests demonstrate, 10 ml. of the product of this invention is as effective as approximately 1000 ml. of fresh plasma. Of even greater importance, patients who have become sensitized to whole plasma and who show anaphylactoid reactions to the injection of plasma tolerate the injection of the purified coagulation components of this invention with no reactions whatsoever. Naturally, tests comparing the product of this invention with plasma cannot be made on these patients.

By various means, purified plasma thromboplastin component free of prothrombin have been prepared. The *in vivo* administration of prothrombin-free plasma thromboplastin component has not fully benefited haemophilia B patients, nor does the administration of prothrombin alone benefit haemophilia B patients. There is no ready explanation for this phenomenon. When our product containing the three purified coagulation components together is administered to patients, the plasma thromboplastin component potency *in vivo* is not related to its prothrombin content or to its proconvertin content but appears to be related to its plasma thromboplastin component content plus its prothrombin content. The effect of injection of the product on the *in vivo* prothrombin plus proconvertin level is far in excess of that predictable from the amount of these factors injected. We are unable to explain this discrepancy.

In proconvertin deficient patients, our product has been found to be just as effective in remedying the coagulation defect as in plasma thromboplastin component deficient or haemophilia B patients. The administration of over 1000 ml. of fresh plasma to such patients does not significantly alter their blood prothrombin level, or proconvertin level, or their combined prothrombin plus proconvertin level, but does temporarily remedy their coagulation defect. However, the administration of our product containing the admixed three coagulation components results in an immediate and prolonged rise in both the proconvertin level and the prothrombin plus proconvertin level.

In both plasma thromboplastin component deficient patients and proconvertin deficient patients the administration of fresh plasma does not measurably increase the *in vivo* plasma prothrombin level. However, the administration of the purified product of this invention does increase the *in vivo* prothrombin level from 60 per cent of normal up to 100 per cent of normal. Furthermore, the co-administration of prothrombin appears necessary for the plasma thromboplastin component to be fully effective clinically.

Summary.

The process herein described effects the simultaneous recovery and purification of three inter-related coagulation components. Of great importance is the degree of concentration which is achieved. On a protein basis, the coagulation components are concentrated 400-fold. In other words, 99.75 per cent of the total plasma protein is

removed and is available for the preparation of other known, clinically useful plasma fractions. The antihaemophilia A factor is spared and is available for concentration and purification whenever a suitable process is discovered. The high degree of purity in which the three coagulation components is obtained is also of paramount importance clinically. The removal of 99.75 per cent of unneeded plasma protein renders the product suitable for the treatment of the congenital coagulation defects in patients who have become sensitized to whole plasma, or who have become refractory to treatment with whole plasma, and thus offers these patients a means of renewing maintenance therapy.

The concentration in volume is also of great importance clinically. Aside from the obvious convenience, economy, and safety of a purified concentrate, it is very easy to achieve an effective *in vivo* blood level of the coagulation components by injection of 10 ml. in a few minutes, whereas it is very difficult to achieve an effective *in vivo* blood level by injecting 1000 ml. of plasma over a period of 4 to 5 hours. Both the time and the dilution hinder the rapid correction of the coagulation defect.

Except for the provision of a highly purified and concentrated fibrinogen, this is the first time that any coagulation components have been separated and made available in a form far more useful clinically than the plasma from which they were derived. Furthermore, as pointed out in the description of clinical tests, the coagulation components of this invention are far more effective when administered simultaneously than when only one of the components is administered to correct a specific defect.

It should be pointed out that the product of this invention is devoid of thrombin, fibrinogen, thromboplastinogen (anti-haemophilia A factor) and accelerator globulin. Consequently, the product is of no value in the treatment of bleeding episodes due to deficiencies of fibrinogen, anti-haemophilia A factor (thromboplastinogen) or accelerator globulin.

WHAT WE CLAIM IS:—

1. A derivative of human blood comprising: plasma thromboplastin component, proconvertin and prothrombin, which mixture is free from other thromboplastin precursors.

2. A mixture of blood coagulation components comprising: plasma thromboplastin component, proconcertin and prothrombin, which mixture is free other thromboplastin precursors.

3. Derivative according to Claim 1 wherein the plasma thromboplastin component, proconvertin and prothrombin are present in substantially the same proportions as they occur in the human blood.

4. The process of collectively recovering the proteins; plasma thromboplastin component, proconvertin and prothrombin from non-citrated human plasma, comprising: mixing said plasma with an adsorbing agent such as barium sulphate, separating said adsorbing agent and its adsorbed proteins from the plasma, eluting the adsorbed proteins from said adsorbing agent with a sodium citrate solution, adjusting the *p*H of the solution to about 3.2, precipitating the proteins with a lower aliphatic alcohol, and collecting the resulting precipitate.

5. The process according to Claim 5 wherein said lower aliphatic alcohol is ethanol.

6. The process of fractionating the blood plasma proteins adsorbable on barium sulphate substantially as herein described with particular reference to the example.

7. The process of collectively recovering proteins from non-citrated human plasma substantially as herein described with particular reference to the example.

8. The product of the process of either Claim 4 or 5.

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Chartered Patent Agents,
Agents for the Applicants.

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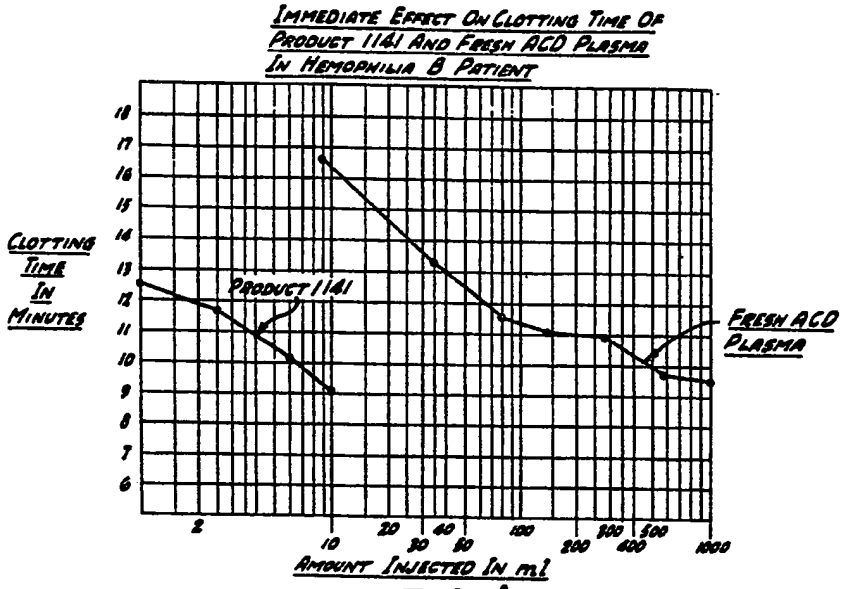
IMMEDIATE EFFECT ON CLOTTING TIME OF PRODUCT 1141 AND FRESH ACD PLASMA IN HEMOPHILIA B PATIENT
CLOTTING TIME IN MINUTES
18
17
16
15
14
13
12
11
10
9
8
7
6
PRODUCT 1141
FRESH ACD PLASMA
2
10
20
30
40
60
100
200
300
400
600
1000
AMOUNT INJECTED IN ml

FIG-1

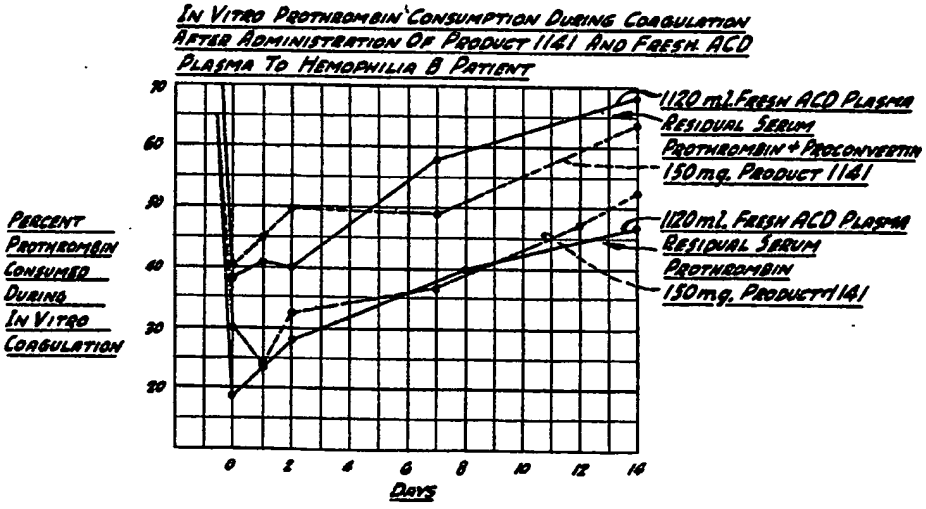
IN VITRO PROTHROMBIN CONSUMPTION DURING COAGULATION AFTER ADMINISTRATION OF PRODUCT 1141 AND FRESH ACD PLASMA TO HEMOPHILIA B PATIENT
PERCENT PROTHROMBIN CONSUMED DURING IN VITRO COAGULATION
70
60
50
40
30
20
1120 ml. FRESH ACD PLASMA
RESIDUAL SERUM
PROTHROMBIN + PROCONVERTIN
150 mg. PRODUCT 1141
1120 ml. FRESH ACD PLASMA
RESIDUAL SERUM
PROTHROMBIN
150 mg. PRODUCT 1141
0
2
4
6
8
10
12
14
DAYS

FIG-2

872,536 COMPLETE SPECIFICATION
2 SHEETS

This drawing is a reproduction of the Original on a reduced scale.
SHEETS 1 & 2

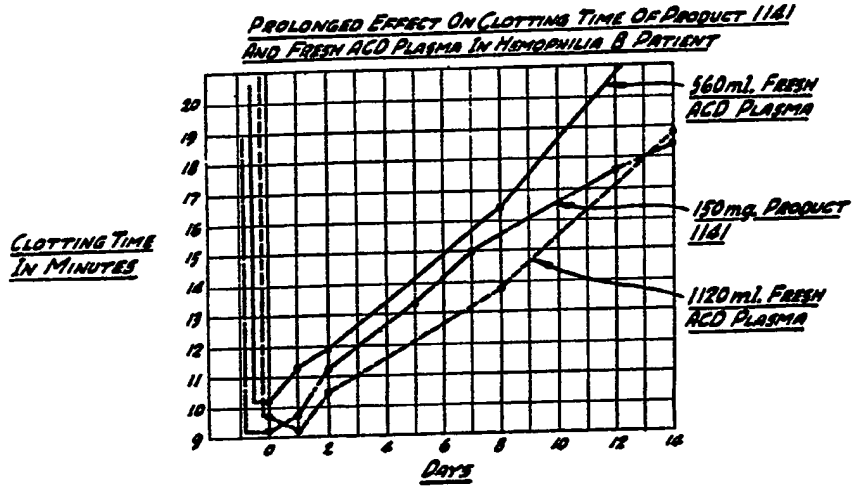

FIG-3

872,536 COMPLETE SPECIFICATION
2 SHEETS This drawing is a reproduction of the Original on a reduced scale.
SHEETS 1 & 2

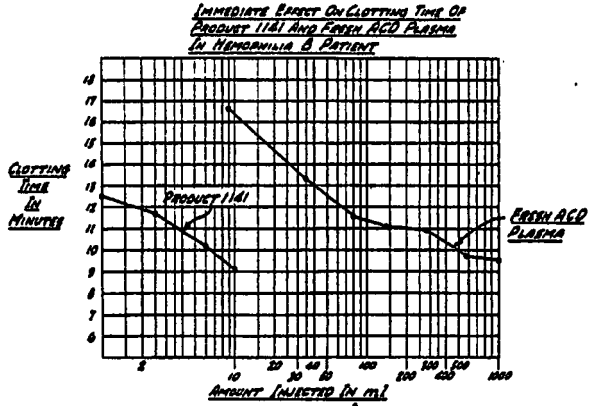

FIG-1

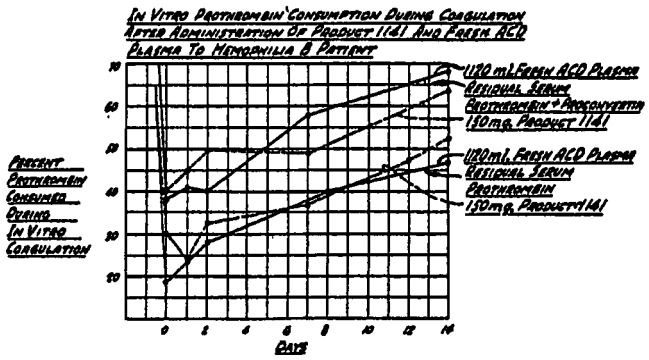

FIG-2

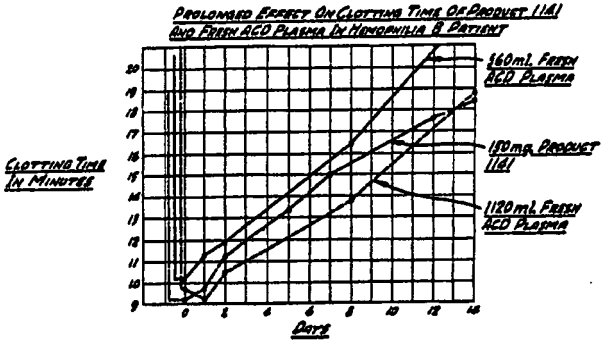

FIG-3